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International Biodeterioration & Biodegradation 45 (2000) 175–181

INTERNATIONAL
BIODETERIORATION &
BIODEGRADATION

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Potential for cell culture techniques as a wildlife management tool for screening primary repellents

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Abstract

The identification of new chemical repellents for wildlife damage management is impeded by the need to perform cumbersome and expensive behavioral tests. Here, we report the development of in vitro cell culture methods to increase the speed and efficiency of repellent screening while reducing costs, as well as the number of animals necessary for research. Our methods exploit the fact that effective primary repellents depend on the stimulation of pain receptors. We cultured trigeminal nociceptors (pain receptors) from Norway rat (*Rattus norvegicus*, laboratory strain), white leghorn chicken (*Gallus gallus*), coyote (*Canis latrans*), white-tailed deer (*Odocoileus virginianus*) and Canada goose (*Branta canadensis*), and then applied digital fluorescence microscopy to measure changes in intracellular calcium (an index of cellular activation) in response to applications of known and effective repellents. Capsaicin, bradykinin and acetylcholine were more effective stimuli for rat, coyote, and deer neurons than cells from chicken. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Chicken; Canada goose; Coyote; Deer; Neuron; Rat; Repellent; Trigeminal

1. Introduction

Both the general public and professional wildlife managers are expressing interest in new repellent chemicals for non-lethal wildlife control (Mason, 1997). Despite this increasing interest, few repellent compounds are available for use, and the number of such compounds has been decreasing (Clark, 1998a). This decrease in commercially available products reflects reregistration requirements imposed by the U.S. Environmental Protection Agency under the Federal Insecticide, Fungicide, and Rodenticide Act, and the fact that new repellents are expensive to develop, register and commercialize (Mason and Clark, 1992, 1997; Clark, 1998a). The only new avian primary

repellent developed for use in the United States within the past decade is methyl anthranilate (commercially available as ReJex-It AG-36, R.J. Advantage, Inc., Cincinnati, OH), a substance that is commonly used in human foods and flavorings. To reduce development costs, several laboratories (including our own) have developed behavioral screening procedures to evaluate commercially available substances that are chemically similar to methyl anthranilate (e.g., acetophenones (Mason et al., 1991a,b; Clark and Shah, 1991); aromatic acetates (Clark and Aronov, 1999); benzoates (Clark et al., 1991; Clark and Shah, 1994); cinnamic acids (Crocker and Perry, 1990); coniferyl benzoates (Jakubas and Mason, 1991; Jakubas et al., 1992); vanillylates (Shah et al., 1991)). These efforts, while sometimes effective, have been expensive and slow to produce new candidate products. In addition, because the screening tests have been behavioral, large numbers of animals have been required to achieve statistically reliable results.

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Effective wildlife repellents fall into two broad categories: primary and secondary repellents (Rogers, 1974). Secondary repellents are chemicals that produce illness after being ingested to which the animal subsequently learns to avoid associated cues, e.g., tastes, smells and visual cues. Primary repellents are compounds that are congenitally avoided because they are perceived as noxious. Primary repellents are typically irritants that activate polymodal nociceptors (pain receptors) in the somatosensory system (Alarie, 1990; Silver et al., 1991; Szolcsanyi, 1993; Mason et al., 1989; Clark, 1995). Taste and smell are rarely, if ever, important for the sustained aversive effects of primary repellents (Mason et al., 1989; Clark, 1996, 1998b).

Recently, we adapted standard cell culture techniques to examine the in vitro response of polymodal nociceptors to candidate repellent compounds (Bryant, 1997). The objective of this study was to determine the feasibility of using the cell culture approach as a method to screen for primary repellents. To test the general applicability of our methods, we examined cells derived from both mammals and birds, including several species of considerable importance to wildlife damage management.

2. Materials and methods

2.1. Subjects

Norway rats, *Rattus norvegicus*; white leghorn chicken, *Gallus gallus domesticus*; coyote, *Canis latrans*; white-tailed deer, *Odocoileus virginianus*; and Canada goose, *Branta canadensis* were used as source material. Norway rat pups were obtained 2 days post-partum from pregnant dams (Sprague–Dawley strain, 250–300 g) purchased from Charles River, Wilmington, DE. Embryonated white leghorn chicken eggs were obtained from Truslow Farms, Chesterton, MD. Chicken embryos were removed from eggs at 17 days post-fertilization. Embryonated Canada goose eggs were obtained from USDA-APHIS-Wildlife Services (WS) personnel who collected the eggs during goose management activities near Harrisburg, PA. After delivery to the laboratory, the goose eggs were incubated at 37.4°C until an age of 19–23 days, as determined by floatation technique (Westerkov, 1950). Deer tissues were collected from one adult female and one male yearling euthanized by gunshot as part of deer management activities by WS personnel at Lehigh Valley International Airport, Allentown, PA (May, 1998), two female yearlings collected for the same reason at the National Aeronautics and Space Administration Research Facility, Sandusky, OH (July, 1998), and two yearlings, one male and one female, collected during damage management activities at the Philadelphia In-

ternational Airport, Philadelphia, PA (December, 1998). Coyote tissues were obtained from 48-week old male coyote pups shipped to the laboratory from the Predation Ecology and Behavioral Applications Field Station of the USDA-APHIS-WS-National Wildlife Research Center, Logan, UT.

2.2. Tissue collection

We obtained ganglia from all species within 5–30 min of euthanasia. Deer were sacrificed by gunshot, and then bled by jugular puncture to relieve pressure that might have interfered with cranial dissection. We swabbed the cranium with 70% ethanol to sterilize the surgical field, removed the skin, sterilized again with 70% ethanol and then removed the top of the skull with a portable circular saw. Ganglia were harvested as described below.

Coyote pups and rat pups were sacrificed by pentobarbital overdose. As with deer, the surgical field was sterilized with 70% ethanol, and then the skin and top of the skull were removed. We collected avian ganglia from embryos we removed from ethanol sterilized eggs.

Trigeminal ganglia are located in the middle cranial fossa and project to the brainstem. For this reason, we exposed the brains in donor animals, and then severed the optic and olfactory nerves so that the brains could be reflected to reveal the trigeminal ganglia. We used a sterile scalpel to cut the central and peripheral connections to the ganglia and to split the surrounding fascia. We removed the exposed neural tissue and transferred it to a container of sterile Hanks buffered saline solution (HBSS). We added penicillin (50 units/ml) and streptomycin (50 µg/ml) to HBSS to prevent bacterial infection of cultured deer and coyote tissues.

2.3. Culture of neurons

General methods of cell culture followed those outlined in Banker and Goslin (1991). Within 5 min of sacrifice, we removed ganglia into HBSS, and minced the neural tissues to 1 mm pieces. We incubated these pieces in an aqueous solution containing proteolytic enzymes at 37°C with mild agitation for 25 min to release individual cells. For deer and coyote ganglia, the proteolytic solution contained 0.1% trypsin, 0.1% collagenase and 0.01% DNase. For rats, chicken, and geese, the solution only contained 0.1% collagenase. We added trypsin and DNase to the solution used with deer and coyote tissues because of the more advanced age of the donor animals and consequently, the presence of more connective tissue.

After the enzyme treatment, tissues were triturated with a fire-polished Pasteur pipette and then passed through a 90 µm filter to produce a suspension of glial

cells, fibroblasts, and neurons. For coyotes, deer, and rats, we preplated 6 ml of suspension onto poly-L-lysine coated 60 mm plastic culture dishes that were vibrated for 10 s every 10 min for 2 h at 37°C in a 5% CO₂ atmosphere. This produces a supernatant enriched in neurons because glia and fibroblasts attach more readily to the dishes relative to neurons. We used an aquarium air pump as the source of vibration. We did not preplate the suspensions we prepared from goose and chicken donors because embryonic tissues contain a higher proportion of neurons relative to glia and fibroblasts, collectively.

We plated the resulting neuron-rich supernatants from coyote and rat in DMEM:F12 medium containing 10% fetal bovine serum, and 3% glutamine onto #1 glass cover slips coated with poly-L-lysine (100 ng/ml) and laminin (5 µg/ml). We plated preparations from deer, chicken, and goose in Liebovitz L15 medium with horse serum, containing 10% glucose, 3% L-glutamine, and glutathione and ascorbic acid. We supplemented this medium with a mixture of vitamins, amino acids, and other cofactors (Baumann, 1993). As for coyotes and rats, we used #1 glass cover slips with deer, geese, and chicken, prepared as described above. After 18 h, we refreshed the plating medium with new solution containing 100 ng/ml of nerve growth factor (mouse 7S, Sigma, St. Louis, MO). Depending on the medium, we incubated neurons in a 5% CO₂ (DMEM/F12) or ambient air (Liebovitz L15) atmosphere. We incubated avian neurons at 38°C and mammalian neurons at 37°C for 48–72 h. We microscopically examined plated mediums, and found that in all cases, surviving neurons developed extensive neurite outgrowth.

2.4. Measurement of neural response

We used digital fluorescence calcium imaging to determine intracellular calcium $[Ca^{2+}]_i$ in 2–4-day old cultured neurons (Gryniewicz et al., 1985; Restrepo et al., 1995). Briefly, we loaded neurons by incubation with 5 µM fura-2/AM and 80 µg/ml Pluronic-127 (60 µg/ml Pluronic-127 for chicken neurons) in culture medium at 24°C for 1 h. We illuminated fields of cells at 340 nm (Ca^{2+} sensitive) and 360 nm (Ca^{2+} insensitive) with a grating monochromator to enable ratio-metric measurement. We imaged fluorescing neurons with a cooled integrating charge-coupled device (CCD) camera. We calculated intracellular calcium concentrations ($[Ca^{2+}]_i$) from the fluorescence ratio using the equation $[Ca^{2+}]_i = K_d(F_o/F_s)(R - R_{min})(R_{max} - R)$, where K_d is the calcium dissociation constant for fura-2 (224 nm), and R_{min} and R_{max} are the fluorescence ratios at low $[Ca^{2+}]$ (1 mM EDTA) and high $[Ca^{2+}]$ (5 mM Ca^{2+}), respectively, F_o/F_s is the ratio of fluorescence emitted by the fully bound dye to the fluor-

escence emitted by the dye in the absence of Ca^{2+} at 360 nm. The fluorescence ratio indicating $[Ca^{2+}]_i$ was displayed on a monitor as a pseudocolor image.

At the beginning of an experiment, we focused on randomly selected isolated neurons that appeared healthy and had good morphological features. We digitally outlined "areas of interest" surrounding each neuron in the digital fluorescent image, and then examined changes in $[Ca^{2+}]_i$ in response to test stimuli delivered to the cells in Ringer's solution. For coyotes, rats, and deer, this solution contained (in mM): 120.0 NaCl, 5.0 KCl, 5.0 CaCl₂, 1.0 MgCl₂, Cl₂, 5.0 HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 10.0% glucose, pH 7.4. For chicken, the solution contained (in mM): 138.3 NaCl, 5.8 KCl, 5.0 CaCl₂, 1.0 MgCl₂, 5.0 HEPES, 10.0% glucose, pH 7.4. Cell autofluorescence was negligible. We detected no appreciable bleaching during continuous 1 h sessions. We made fluorescence determinations every 10 s using UV illumination that lasted for 60–100 ms. Chemical stimuli were flowed into the chamber in which the coverslip was located for 15 s and the chambers were rinsed for 3–4 min between stimuli. In order to characterize the responses of neurons from different species for interspecific comparison, we used a prototypical irritant (capsaicin) and endogenous compounds that cause pain in humans (acetylcholine, bradykinin, histamine, and serotonin). Concentrations are indicated below.

Although goose cells were grown in culture, an insufficient number of cells were available for imaging experiments.

Methyl anthranilate was tested on cultured neurons, however, it was discovered that the compound emits interfering fluorescence when exposed to the wavelength of ultraviolet light used to excite the calcium-sensitive dye, fura-2. Future studies will examine cultured neuronal sensitivity to methyl anthranilate using a dye that does not suffer this interference.

3. Results and discussion

3.1. Culturing neurons

We found that after 1–3 days in culture, neurons from all species showed normal development of neuritic extensions and typical round somatic morphology. Neurons typically continued to grow in vitro for 7–10 days, after which they began to degenerate and die.

We observed that large numbers of neurons only grew when the delay between the death of the donor and the dissociation of ganglionic tissue was short. For this reason, we were least successful in our attempt to culture cells from white-tailed deer. Although ganglionic tissue was removed in most cases

in less than 5 min following death, cell dissociation procedures were sometimes delayed by ≥ 90 min, while chilled tissue samples were transported to the laboratory. None of the samples subjected to these delays produced viable neurite-bearing cells. We were able to successfully culture neurons from deer only when the interval between the death of the donor and the initiation of cell culture was ≤ 15 min. We obtained our highest neuron yields (from coyotes, rats, chicken, and geese) when culture procedures were initiated immediately (within 5–10 min) following removal of ganglia from donors.

The appropriate buffer to be used is determined by practical considerations. DMEM-F12 is a $\text{CO}_2/\text{HCO}_3^-$ -buffered medium that is only useful when neurons can be placed immediately in a 5% CO_2 incubator. We found that rat and coyote neurons developed well in this medium. Liebovitz L15 medium is buffered with HEPES and does not require a CO_2 atmosphere. This medium supported the development of neurons from all species tested.

3.2. Functional responses of trigeminal neurons to prototypical chemical irritants

When primary irritants (i.e., capsaicin) and neurochemicals associated with pain (i.e., acetylcholine, bradykinin, histamine, serotonin) were delivered to cultured trigeminal neurons derived from rats, deer, coyotes, and chicken, we obtained reversible changes in $[\text{Ca}^{2+}]_i$ indicative of cellular activity. These changes were relatively fast. Peak responses occurred within 5–10 s of stimulation and the cell recovered to baseline

activity within 3 min or less, allowing us to conduct multiple tests with each isolated neuron. This type of internal control was important because other studies showed that neurons were specific in their responsiveness to certain chemicals, reflecting differences in receptor populations and threshold sensitivity of individual cells to specific stimuli (Wang et al., 1993; Besou and Perl, 1969).

The heterogeneity in the responsiveness and sensitivity of neurons to chemicals among the species also was apparent in our study. For example, Fig. 1 shows the activity pattern for three neurons derived from a coyote. One neuron responded exclusively to 0.5 μM capsaicin. One neuron responded exclusively to 10 μM histamine. The third neuron responded to both 0.5 μM capsaicin and 10 μM histamine. None of these neurons were responsive to an organic acid solution (pH 6.0), or two additional common neurochemicals coding for pain (1 μM bradykinin and 10 μM serotonin). Fig. 2 is an example of the responsiveness of an isolated deer neuron. In this case the neuron was insensitive to 10 μM acetylcholine, but was responsive to 1 μM bradykinin and 1 μM capsaicin.

Despite the observed variability in the responses of the trigeminal neurons across species, chemicals, and concentrations as exemplified above, some intriguing patterns were apparent. When all viable neurons were considered, irrespective of the identity or concentration of the test chemical, chickens, rats, and deer were found to have different proportions of chemically responsive neurons; 19%, 58%, and 77% for chicken, rat and deer neurons, respectively. In addition, the pattern of responsiveness differed between species

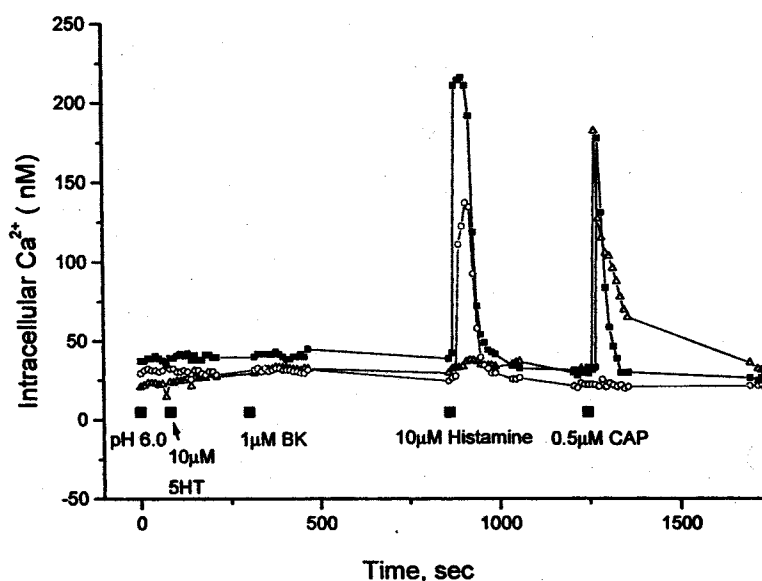


Fig. 1. $[\text{Ca}^{2+}]_i$ response of three coyote trigeminal neurons to a pH 6.0 solution, 10 μM serotonin (5HT), 1 μM bradykinin (BK), 10 μM histamine, and 500 nM capsaicin (CAP).

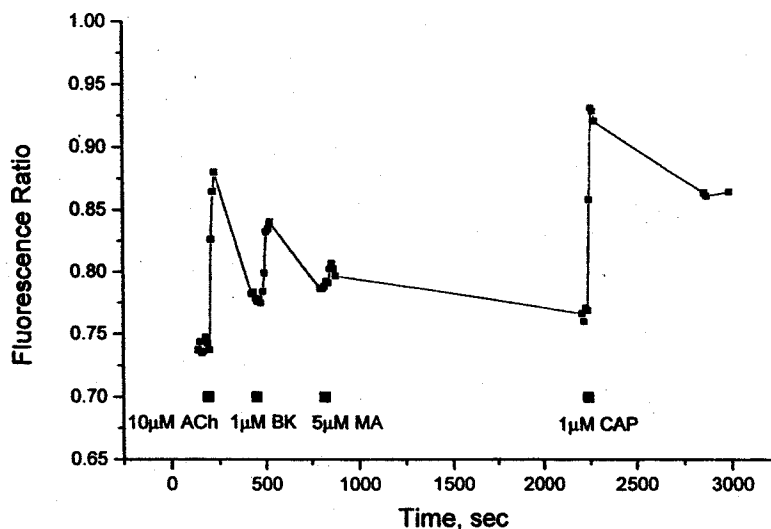


Fig. 2. $[Ca^{2+}]_i$ response of a deer trigeminal neuron to 10 μ M acetylcholine (ACh), 1 μ M bradykinin (BK), 5 μ M methyl anthranilate (MA) and 1 μ M capsaicin (CAP). Note the lack of response to MA.

(Table 1). For chicken, 12.2% of viable neurons responded to capsaicin, while for rats and deer, a higher proportion of neurons were sensitive to capsaicin, 58.3% and 51.2%, respectively.

It is interesting to note that a proportion of avian neurons responded to capsaicin. This is somewhat surprising, given the lack of behavioral sensitivity to the irritating qualities of capsaicin by birds. These findings are not without behavioral precedent because Mason and Clark (1995) showed that starlings could form a trigeminally mediated conditioned avoidance response to capsaicin for concentrations greater than 5000 ppm. These results suggest that birds have receptors for capsaicin, but that the taxonomic difference with respect to avoidance responding reflects differences in how these taxa centrally process the information.

While these findings agree with the behavioral based generalizations that there are taxonomic differences in responsiveness to nociceptive stimuli (Mason et al., 1991a), the data also suggest that birds are less chemically sensitive than mammals. However, we must point out that the proportion of responsive neurons is only one index of chemosensitivity. The magnitude of the response and the pattern of firing of neuron(s) are also

critical to determining perceptual sensitivity. We do not report on these considerations in this preliminary study, but these factors will be considered in detail in future studies. Furthermore, while capsaicin-sensitive nociceptors are the most important sources of peripheral inputs for chemically-induced pain in mammals (Szolcsanyi et al., 1993), and methyl anthranilate-sensitive nociceptors are important sources of peripheral inputs for chemically-induced pain in birds (Mason et al., 1989; Clark, 1996), other neurons that respond mainly to thermal and tactile stimuli can contribute to the perception of chemical pain (Craig and Bushnell, 1994; Mason et al., 1996; Bryant and Mezine, 1999). We are attempting to identify stimuli that activate these additional types of neurons to increase the efficiency and accuracy of the *in vitro* assay procedures described here.

Finally, capsaicin-sensitive neurons cultured from rats responded identically to neurons cultured from deer and coyotes (Table 1). We speculate that rat neurons may be useful surrogates for neurons obtained from these, and perhaps other species of mammalian wildlife. If this speculation is correct, then *in vitro* screening will greatly enhance the efficiency and economy of repellent development for several reasons. First, it would make the capture and sacrifice of large, expensive, and/or difficult-to-maintain species of wildlife unnecessary. Second, source tissues would be available year-round. Finally, because perinatal tissues are easier to harvest and successfully culture, the accuracy of *in vitro* screening would be higher than the accuracy of behavioral screens because hundreds (rather than tens) of data points could be collected for the calculation of concentration–response curves.

Table 1
Percent of cultured trigeminal neurons responding to irritant stimuli. Numbers in parentheses are the numbers of neurons tested^a

	Rat	Deer	Coyote	Chicken
Capsaicin	58.3 (48)	51.2 (43)	47.0 (17)	12.2 (82)
Acetylcholine	33.3 (3)	39.2 (38)	0 (3)	21.7 (23)
Bradykinin	33.3 (3)	21.1 (38)	30.0 (10)	13.0 (8)

^a Some neurons responded to multiple chemicals.

4. Management implications

Despite the promise of the methods described here, we feel it is important to note that additional data are needed in several areas. First, because neurons were heterogeneous in responding to chemical irritants, we believe additional quantitative studies will be necessary to confirm that rat nociceptors can serve as a generalized model for mammalian nociceptors. Second, a quantitative comparison of chicken neural responses with the responsiveness of receptors cultured from other avian species remains to be done. Third, not all trigeminal neurons are nociceptors, and we speculate that it may be necessary to develop cell selection methods to enrich cultures in polymodal nociceptors.

Apart from increased efficiency and accuracy, in vitro methods have the advantage of minimizing the number of animals used in repellent development research. Simultaneously, these methods will increase the number of compounds that can be evaluated. To illustrate this point, consider the following example. At present, behavioral screening studies require about one week per chemical and as many as 40 animals may be needed as test subjects (Nolte and Mason, 1998). Using five embryos, we may be able to harvest $\geq 150,000$ neurons, half of which are likely to be polymodal nociceptors. In theory, this number of cells would permit the evaluation of 100 concentrations of a chemical with 750 neurons/concentration. Even if we assume a poor collection efficiency (as might occur under field conditions with adult animals) we could still reasonably expect to harvest as many as 5000 neurons, permitting 100 assays with 50 neurons/concentration. If only manual techniques are used, we could prepare a new cell culture every 3–4 days, and evaluate candidate compounds at a rate of 2 chemicals/day. At this rate, we could evaluate 10 substances every 5 days with far greater statistical power and precision concerning mechanism than currently possible using behavioral tests. If automated methods using robotic techniques (Gonzalez and Negulescu, 1998) were implemented, then the number of compounds screened, as well as the number of data points per compound would dramatically increase.

Broadly, in vitro methods hold great promise for the development of new repellent substances. By increasing the speed and efficiency with which candidate materials can be examined, sufficient data may become available to permit the development of predictive models that permit the design and synthesis of novel repellents (Clark and Shah, 1991, 1994; Clark and Aronov, 1999). Already, this pharmacophore approach is being used by the pharmaceutical and the food and flavor industries to develop new products (Brandt, 1998; Demeter et al., 1998). In addition, we anticipate that cell culture screening will permit expanded testing of

natural products as repellents. The existing evidence suggests that many such compounds may be useful as repellents, and some of these materials may be approved as food and flavor ingredients (e.g., Mason et al., 1989; Mason, 1990; Clark 1998b; Clark and Aronov in press) and for which pesticide registration requirements are likely to be minimal (Clark, 1997). Because of taxonomic differences in irritant effectiveness (Norman et al., 1992), it is possible that materials can be identified using this approach that are repellent to birds but not mammals, and vice-versa.

Acknowledgements

Funding was provided by U.S. Department of Agriculture Cooperative Agreement #12-34-41-0040[CA] between the National Wildlife Research Center and the Monell Chemical Senses Center. A. Montoney and T. Tomsa, Wildlife Services, provided fresh deer tissue. Drs. M.W. Fall and J. Rhyan provided valuable comments on an earlier manuscript draft.

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